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Molecular Identification and Genetic Diversity of *Acropora hyacinthus* from Boo and Deer Island, Raja Ampat, West Papua

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Abstract Indonesia lies at the centre of biodiversity for corals. However, the reefs suffered from extensive human exploitation. Marine Protected Areas is thought to be best solution to protect coral reefs ecosystem. Understanding genetic diversity is crucial for effective management of the MPAs, however genetic diversity is rarely been corporate in designing an MPA. Moreover, many MPAs are uneffectively manage due to poor designated and demarcated. Raja Ampat which is located in western tip of West Papua, was designated as a park to mitigate threats and protect the valuable marine resources. Scleractinian corals in the genus *Acropora* are among the most dominant distributed in Raja Ampat waters, including the species of *Acropora hyacinthus*. The research aimed to analyze genetic diversity and to describe the kinship relationship of *Acropora hyacinthus* between 2 populations: Boo Island and Deer Island, Raja Ampat. Genetic marker Cytochrome Oxidase I (CO I) of the mitochondrial genome DNA (mtDNA) was used to analyze genetic diversity. Reconstruction of phylogenetic tree and genetic diversity were made by using software MEGA 5.05 (Molecular Evolutionary Genetics Analysis). The results of this research indicate corals *A. hyacinthus* from Boo Island and Deer Island Raja Ampat are in the low category of genetic diversity and overall had a close genetic relationship of kinship. This is likely due to the small size of the population and few numbers of samples that may not represent the population.

1. Introduction

Indonesia lies at the centre of biodiversity for corals [1]. Though the reefs support life of more than 67.500 villages, however it suffered from extensive human exploitation [2]. The occurrence of bleaching events [3] coupled with emergence of coral diseases [4] even in remote area [5] made reef's condition rapidly declining. Therefore, there is an urgent need for reefs protection.

Marine Protected Area (MPA) is thought to be one of the best strategies for protecting biological diversity, increase fisheries yields, and protect particularly vulnerable life stages of marine species [6].



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For ensure protection, it is important to understand the genetic connectivity among reefs and identify designated protected areas that are self-sustaining and possibly export young propagules to the adjacent reefs[7].

Raja Ampat is located in the heart of the 'Coral Triangle', an area encompassing Malaysia, Philippines, Solomon, Timor Leste, Papua New Guinea and Indonesia, which has the highest coral diversity in the world. The archipelago is comprised of several large mountainous islands including Waigeo, Batanta, Salawati and Misool, and hundreds of small islands such as Kofiau, Gam, Fam and Ayau. The land and surrounding sea occupy approximately 43.000 km². While many of these are uninhabited, most of the population (~43,435) lives on 34 islands in the archipelago [8]. Almost all of the populations depend on their marine resources to support their livelihood [9]. Raja Ampat was designated as Marine Protected Areas (MPA) in order to mitigate threats and protect valuable marine resources. The MPA was established under Government Decree No. 27/2008 which protect more than million hectares of coastal and marine in Raja Ampat areas.

Reefs were generally in very good condition compared to most areas of Indonesia with high live coral diversity and minimal stress due to natural phenomenon such as cyclones, predation (i.e. crown-of-thorns starfish), and freshwater runoff [8]. However, 17% of reefs were considered to be in poor condition, mostly because of high levels of silting and the increase explosive use for fishing. Marine resources in Raja Ampat are also under threat from increasing frequency of disturbances associated with climate change (i.e. coral bleaching).

Documenting the diversity of marine life is challenging because many species are cryptic, small, and rare, and belong to poorly known groups [10]. A genetic identification system would be particularly useful for such organisms, especially whose identification may be uncertain due to phenotypic plasticity and lack of identification tools at early stages of life [11]. DNA barcoding has become an attractive method to identify species over a wide taxonomic range [12]. Cytochrome c oxidase subunit I (COI) has been proposed as the main barcoding gene for metazoans [11]. Though was reported as rapid, inexpensive and need little expertise, however, the method was thought controversial since it simplified various taxonomic characters into one single character of identification only [13]. Yet the method was reported can readily discriminate among closely related species across most animal phyla. The method was successfully documented enormous diversity of various cryptic animals that only 10.9% of the sequences were matched to reference barcodes in public databases [10]. However, it was suggested that DNA barcode was not appropriate for most scleractinian corals because this region evolves very slowly in these organisms and consequently both inter- and intra-specific variation are extremely low [14]. In contrast, DNA barcode was successfully revealed the taxonomy of *Stylophora pistillata* by examining COI divergence and phylogeny of the coral. By analyzing Cytochrome Oxidase I sequences, from 241 samples across 4 different geographic ranges, showed this taxon in fact comprises four deeply divergent clades [15].

Acropora, differ from all other extant coral genera has unique form of polyp which formed by one or more central or axial corallites. The axial corallites bud the radial corallites as the coral grow [16]. Species of *Acropora* are also highly polymorphic and have always carried problems of identification [17]. The genera also plays a dominant role in the species composition and abundance of many Indonesian reefs [18]. *Scleractinian* corals in the genus *Acropora* are among the most dominant distributed genera in Raja Ampat waters. This publication aims to determine the connectivity patterns and genetic diversity of *Acropora hyacinthus* of Raja Ampat in order to understand the genetic diversity of the coral to help conservation program of the MPA.

2. Materials and Methods

Sample collections

Acropora hyacinthus was collected from core zone area of Kofiau Marine Park Area. Since sampling was conducted at MPAs area, prior the sampling, a letter of sampling permission was obtained from the Raja Ampat Bureau of National Marine Park (Simaksi, BTN). Samples were taken from the core zone of Kofiau District. Two islands were chosen as sampling location namely Deer Island (-1.155234, 129.846921) and Boo Island (-1.175641, 129.337620). *A. hyacinthus* at the sampling location showed various colony shape, though most of the colony has tabulate form (Fig.1). Twenty fragments were obtained from each island from each sampling location at the two islands.

Fragments are cut from the middle branches of the coral colony using cutting pliers. The length of fragments approximately 1-3 cm. Fragment were then put inside a labelled ziplock plastic. After collection, fragments were removed and put into 15 ml falcon tube and preserved in 95% ethanol. Fragments are then transported to Integrated Lab (CORESDU) Diponegoro University. To avoid to sample clone, distance of one colony to another colony at least 1 m. Both species are capable to reproduce through fragmentation since the colony shape is branching. Samples are preserved until used.

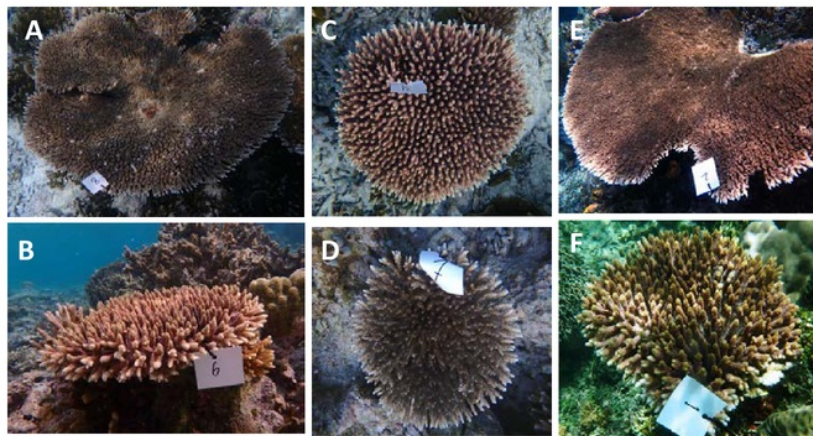


Figure 1. Various type of *A. hyacinthus* collected from Boo and Deer Island, Kofiau District, Raja Ampat. A, B, C, dan D, *A. hyacinthus* collected from Boo Island; E dan D, *A. hyacinthus* from Deer Island

DNA Extraction

A quick and simple DNA extraction method with the resin, Chelex 100 (BioRad) is done. A 0.2mm section of tissues was scrapped using sterile spatula and place into 1.5 ml tubes filled with 300uL 10% Chelex. Vortexed samples in Chelex slurry for 10-15 seconds. Spinned samples briefly (10-15 sec) at high speed in a microcentrifuge. Incubated samples for 20 minutes at 95°C. Vortexed samples again for 10-15 seconds. Spinned tubes again at high speed in microcentrifuge to ensure that all contents are in the bottom of the microcentrifuge tube. Supernatant was directly used for PCR amplification. DNA concentration was then quantified using Nanodrop with spectral length on 260/280 μm and 260/230 μm .

PCR Amplification

Universal primer of DNA barcode were used for this research, there were LCOI1490: 5'-CAAATCATAAAGATATTGG-3' as forward primer and reverse primer : HCOI2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' [19]. PCR was performed using the following thermal

cycle: 1 cycle of 95 °C for 2 min; 40 cycles of 95 °C for 30 s, 47 °C for 90 s, and 72 °C for 90 s. The amplification reaction used 2.5 µL template DNA, 12.5 µL Promega kit, HCOI primer 2 µL, LCOI primer 2 µL and 16 µL distilled water, in total 25 µL volume. The PCR products will be electrophoresed in a 1% agarose (FMC Bioproduct, Rockland, ME, USA) gel in 1x TAE buffer to assess the yield. The PCR products were then genotype using ABI PRISM 3730xl Genetic Analyzer used standard protocol developed by the manufacturer.

Sequencing and Alignment

Sequencing was conducted at Genetika Science Indonesia. Prominent bands of PCR samples were sent to the company. According to the company protocol, sequencing were conducted using Big Dye Terminator v.3.1 Cycle Sequencing Kit follows the PCR cycle sequen. The formula of sequen procedure were as follows : 8 µl big dye, DNA template, 3.2 pmol primer, add millipore water into the final volume 20 µl. While sequencing cycles are as follows: initial denaturation at 95 °C for 5 minutes, then following by 50 cycles of (denaturation at 95 °C for 30 second; annealing at 48 °C for 10 second; and extension at 60 °C for 40 minutes). The results were then kept in 4 °C until purification.

Purification was done by purify the amplified DNA using 5 µl EDTA 100 mM into PCR products. And then was added 60 µl of 100% ethanol. Mixed samples were then be homogenized and were incubated at room temperature for 15 minutes. Samples were then centrifuged at 2000 – 3000 rpm for 30 minutes. Then added 60 µl of 70% ethanol into the samples tube. Samples were then be centrifuged again at 4 °C with 1650 rpm speed for 15 minutes. Purified DNA then were sequenced using ABI PRISM 3730xl Genetic Analyzer following the protocol available from the Applied Biosystems, USA. The most prominent band of purified PCR products are used as sequencing samples.

The sequenced results were then alignment and were analyzed using BLAST (*Basic Local Alignment Search Tool*) based on genetic data base of NCBI (National Institute for Health, USA (www.ncbi.nlm.nih.gov)). Phylogenetic tree was constructed using MEGA5. *Neighbor Joining Tree* was used to construct a tree to analyse genetic distance. An outgroup was used when constructed the tree. Genetic diversity was observed using DNAsp software [20] and Arlequin: <http://cmpg.unibe.ch/software/arlequin35/Arlequin35.html>.

Results and Discussion

In total, there were 25 sequenced resulted from Deer and Boo Island samples. The resulting Oxidase I (COI) of the mitochondrial genome DNA (mtDNA) sequences corresponding to the genotype were analyzed for homologies with sequences in the data base using BLAST searching. BLAST analysis showed that all 25 sequences were closely related to *Acropora hyacinthus* under 99% similarity (Table 1). The accession numbers of COI mtDNA of the sources that used as comparison was presented as well in Table 1. All nucleotide sequences of the COI were then deposited to GeneBank. All nucleotide sequences have been deposited in the GeneBank database under accession number LC187992 - LC188016 (Table 1).

Table 1. BLAST analysis of Deer and Boo Island samples

No.	Sample code	Length of sequence	Closest relative in GenBank (deposited Accession Number)	Homology	Accession Number
1.	PD Ah 7	707 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188011	99%	KF448531.1

2.	PD Ah 6	714 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188010	99%	KF448531.1
3.	PD Ah 4	707 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188009	99%	KF448531.1
4.	PD Ah 3	716 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188008	99%	KF448531.1
5.	PD Ah 20	712 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188016	99%	KF448531.1
6.	PD Ah 16	705 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188015	99%	KF448531.1
7.	PD Ah 15	707 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188014	99%	KF448531.1
8.	PD Ah 13	720 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188013	99%	KF448531.1
9.	PD Ah 12	668 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188012	99%	KF448531.1
10.	PD Ah 1	705 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188007	99%	KF448531.1
11.	PB Ah 9	690 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC187998	99%	KF448531.1
12.	PB Ah 6	728 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC187997	99%	KF448531.1
13.	PB Ah 5	707 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC187996	99%	KF448531.1
14.	PB Ah 4	694 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC187995	99%	KF448531.1
15.	PB Ah 3	704 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC187994	99%	KF448531.1
16.	PB Ah 20	706 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188006	99%	KF448531.1
17.	PB Ah 2	699 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC187993	99%	KF448531.1

18.	PB Ah 19	707 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188005	99%	KF448531.1
19.	PB Ah 18	701 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188004	99%	KF448531.1
20.	PB Ah 16	706 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188003	99%	KF448531.1
21.	PB Ah 15	706 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188002	99%	KF448531.1
22.	PB Ah 14	711 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188001	99%	KF448531.1
23.	PB Ah 12	710 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC188000	99%	KF448531.1
24.	PB Ah 10	707 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC187999	99%	KF448531.1
25.	PB Ah 1	706 bp	<i>Acropora hyacinthus</i> mitochondrion, complete genome LC187992	99%	KF448531.1

Phylogenetic Tree

Reconstruction of phylogenetic tree was done using PAUP* 4.0 (*Phylogenetic Analysis Using Parsimony*) [21] with *parsimony* method and 1000 times bootstrap. Kinship relationship was observed using *TreeView* program.

Phylogenetic construction based on COI sequences found 6 distinct cluster of *A. hyacinthus*. *Outgroup* using DNA data base of *Montipora* sp. Phylogenetic tree of *A. hyacinthus* was shown at Figure 2.

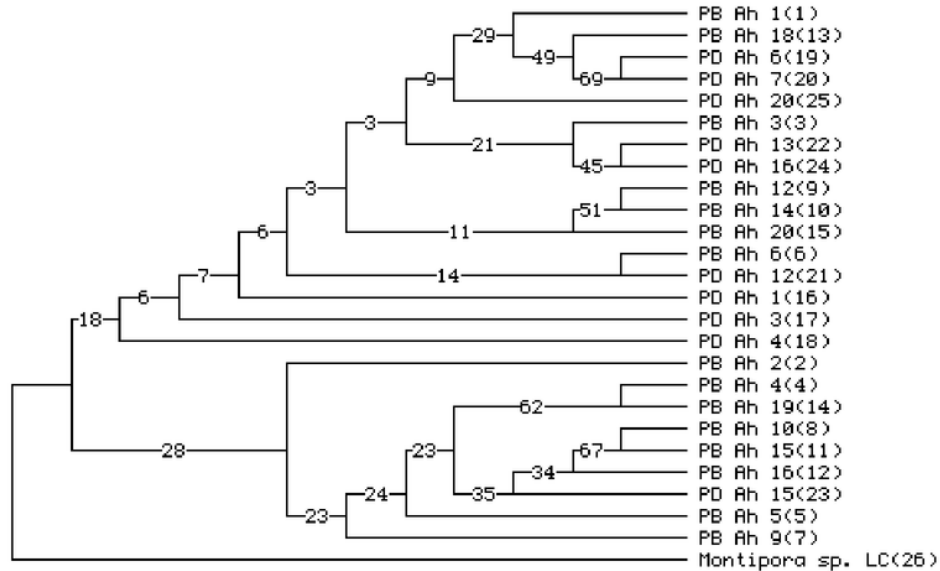


Figure 2. Phylogenetic tree construction of *Acroporahyacinthus* collected from Boo and Deer Island, Kofiau District, Raja Ampat. There 6 different clades observed from the tree. *Montipora* sp was use as outgroup; B, represents Boo Isl.; D, represents Deer Isl.

Clade 1 was comprised of mix samples collected from Boo and Deer Island (PB Ah 1, PB Ah 4, PB Ah 5, PB Ah 6, PB Ah 9, PB Ah 10, PB Ah 12, PB Ah 14, PB Ah 15, PB Ah 16, PB Ah 18, PB Ah 19, PB Ah 20, PD Ah 1, PD Ah 4, PD Ah 7, PD Ah 13, PD Ah 15, PD Ah 16, and PD Ah 20); clade 2, 3, 4 5, and 6 comprised only one samples (Table 2).

When comparison was made with *A. hyacinthus* population from Karimunjawa Archipelago, similar result was obtained. *A. hyacinthus* of Karimunjawa Archipelago was grouped into 5 different clades [22]. Samples of *A. hyacinthus* from Kofiau District was collected from Deer Island and Boo Island that was separated by more than 100 km, while samples from Karimunjawa Archipelago was collected from two different cluster islands namely Seruni, Sambangan and Genting cluster and Cilik and Menjangan Kecil cluster islands that separate by most far 60 km only. However, the Menjangan Kecil Island and Cilik Island was set behind the main Karimunjawa Island that face of open sea while Seruni, Sambangan and Genting was set inside lagoonal area of Karimunjawa main island.

Analysis of Genetic Diversity

Genetic diversity of *A. hyacinthus* was observed using DNAsp [20]. Based on statistic analysis of genetic diversity of 25 samples showed 6 haplotypes. Distribution of haplotypes was shown at Table 3. Estimation of haplotype diversity value of *A. hyacinthus* was 0,3667. When haplotype diversity was observed from each island it was showed much greater value. Haplotype diversity of *A. hyacinthus* collected from Boo Island was 0,9238 (n=15) while haplotype diversity of *A. hyacinthus* collected from Deer Island was 0,9333 (n=10).

Tabel 2. Haplotype diversity distribution of *Acropora hyacinthus* from Boo and Deer Island, Kofiau District, Raja Ampat.

No	Haplotype	Number of samples	Samples code
1.	Haplotype 1	20	PB Ah 1, PB Ah 4, PB Ah 5, PB Ah 6, PB Ah 9, PB Ah 10, PB Ah 12, PB Ah 14, PB Ah 15, PB Ah 16, PB Ah 18, PB Ah 19, PB Ah 20, PD Ah 1, PD Ah 4, PD Ah 7, PD Ah 13, PD Ah 15, PD Ah 16, PD Ah 20
2.	Haplotype 2	1	PB Ah 2
3.	Haplotype 3	1	PB Ah 3
4.	Haplotype 4	1	PD Ah 3
5.	Haplotype 5	1	PD Ah 6
6.	Haplotype 6	1	PD Ah 12
Total		25	

Using multi-locus nucleotide sequence data, [23] recently described two, previously unsuspected, cryptic species complexes within the *Acropora* syngameon (i.e., a groups of species connected through genetic exchange). Among the Scleractinia, the genus *Acropora* contains the largest number of species, occurs in all tropical oceans, and dominates most reef habitats [24]. The genus is known for high-levels of shared polymorphism between species, which is due, in part at least, to widespread genetic exchange through introgression ([25]; [26]; [27]; [28]). Distributions of species of the coral genus *Acropora* in the Indonesian archipelago show a duality reminiscent of the Wallace's Line patterns seen in terrestrial animals and plants, rather than the concentric pattern predicted by the "centre of origin" model [29]. The duality is due to an overlap of Indian Ocean species distributions diminishing eastwards and Pacific Ocean species distributions diminishing westwards within the archipelago [29]. Additionally, a large number of species with broad Indo-Pacific distribution, as well as some regional endemics, occur within the archipelago. For instance, an endemic Indonesian species *A. (Acropora) togianensis*, Wallace, 1997, based on its reproductive activity, morphological and genetic analyses was reported belongs to *Isopora* [16]. Thus although Indonesia has a high species number overall, this is due to the presence of a composite fauna with strong regional differences.

The highest level of endemism was seen to occur in the Togian Islands, in Central Sulawesi, a location with a particularly unusual *Acropora* fauna [29]. The endemic species of the Togian Islands were hypothesized to indicate a possible late Tethyan relictual fauna [30], a conclusion which is reassessed here, and the earliest fossil record of the genus is dated at late Paleocene [16]. Because of this, *Acropora* offers an ideal opportunity to follow global colonization through time in detail. If Indonesia were to be regarded as a single entity, (rather than as six separate areas) its diversity would be highest at 91. This record could possibly be challenged by further collecting in the south-western Philippines and other parts of the South China Sea. No single area of Indonesia, however, shows such a high species count. The greatest number of species found in an area of Indonesia is 77, in the Bay of Tomini, Central Sulawesi. This is also the highest species count worldwide. At the same time, the bay is the smallest area included in the study, being the area between the northern and central eastern arms of Sulawesi.

The results of this research indicate that corals *A. hyacinthus* from Boo Island and Deer Island, Raja Ampat are in the low category of genetic diversity and overall had a close genetic relationship of kinship. This is likely due to the small size of the population and few numbers of samples that may not represent the population. Application of more suitable marker for detecting genetic diversity will help to obtain more robust results.

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PAGE 11